COPY

Attorney Docket No. 0923.003/31807

IN THE UNITED STATES
PATENT AND TRADEMARK OFFICE

In the Application of:

de Boer, et al.

Serial No.: 08/070,158

Filed: May 28, 1993

For: "Anti-CD40 Monoclonal Antibodies Capable of Blocking B-Cell Activation" (As Amended)

Group Art Unit: 1806

Examiner: Hutzell, P.

CERTIFICATE OF MAILING

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail, postage prepaid, in an envelope addressed to: Hon. Commissioner of Patents and Trademarks, Washington, D.C. 20231, on this date:

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Donald J. Pochopien

Donald J. Pochopien
Registration No. 32,167
Attorney for Applicant

DECLARATION OF MARK DE BOER UNDER 37 C.F.R. §1.132

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Mark de Boer declare as follows:

1. That I am a co-inventor of the above-identified application along with Leah B. Conroy.

2. That I am a co-author of the publication, de Boer *et al.*, "Generation of Monoclonal Antibodies To Human Lymphocyte Cell Surface Antigens Using Insect Cell Expressing Recombinant Proteins," J. of Immunological Methods, 152 15-23 (1992) (hereinafter "the de Boer publication").

3. That the de Boer publication discloses the anti-CD40 monoclonal antibodies and hybridomas of the above identified patent application, including 5D12, 3C6, and 3A8.

4. That the de Boer publication lists as co-authors Leah Conroy, Hye Yeong Min and Jaap Kwekkeboom.

5. That the co-authors Hye Yeong Min and Jaap Kwekkeboom are not co-inventors of the subject mater disclosed in the above identified application, including the anti-CD40 monoclonal antibodies, such as 5D12, 3C6, and 3A8, or the hybridomas producing the same.

6. That Hye Yeong Min provided technical support but did not make an inventive contribution to the subject matter of the above identified application.

7. That Jaap Kwekkeboom provided technical support but did not make an inventive contribution to the subject matter of the above identified application.

8. That it is standard practice in the scientific community that publications, such as the de Boer publication, acknowledge the names of all persons who made technical contributions to the data presented.

9. That I am also a co-author of the publication, Kwekkeboom *et al.*, "CD40 Plays An Essential Role In The Activation Of Human B Cells By Murine EL4B5 Cells," Immunology, 79 439-444 (1993) (hereinafter "the Kwekkeboom publication.")

10. That the Kwekkeboom publication lists as my co-authors J. Kwekkeboom, J.M. Tager and C. de Groot, none of whom are named as co-inventors of the above-identified application.

11. That the Kwekkeboom publication at page 440, under the heading "Antibodies and hCD40.Hμ fusion protein" cites to its reference "8," *i.e.*, the de Boer publication of paragraph 2 herein, as the source of the anti-CD40 monoclonal antibodies 5D12, 3C6, 3A8, that are used therein, stating:

> Anti-CD40 mAb 5D12, 3C6, and 3A8 were generated by immunizing mice with insect cells expressing recombinant CD40[8].

12. That my co-authors of the Kwekkeboom publication, *i.e.*, Kwekkeboom, Tager and de Groot, are not co-inventors of the anti-CD40 monoclonal antibodies of the present application, or the hybridomas producing these antibodies, or the methods of using these antibodies as disclosed in the specification of the above identified application.

13. That by citing to de Boer *et al.*, as reference "8" of Kwekkeboom, my co-authors on Kwekkeboom (*i.e.*, Kwekkeboom, Tager and de Groot) tacitly admit that they are not co-inventors of the anti-CD40 monoclonal antibodies of the present application or the hybridomas for producing these antibodies.

14. That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified patent application or any patent issuing thereon.

Mark de Boer

Dated: January 19, 1995

PATENT
Attorney Docket No. 0925.003/31807

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: deBoer *et al.*

Serial No.: 08/070,158

Filed: May 28, 1993

For: "Methods Of Blocking B-Cell Activation Using Anti-CD40 Monoclonal Antibodies"

Group Art Unit: 1806

Examiner: Phillip Gambel, Ph.D.

CERTIFICATE OF FACSIMILE

I hereby certify that this paper is being sent via facsimile to: Assistant Commissioner of Patents, Washington, DC 20231, attention Examiner Phillip Gambel, Ph.D. at 703-308-4242 on this date:

11-20-96

Donald J. Pochopien

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Attorney for Applicant(s)

DECLARATION OF PAUL B. SAVEREIDE, Ph.D.

Assistant Commissioner of Patents
Washington, DC 20231

Sir:

I, PAUL B. SAVEREIDE, declare that:

1. My title at Chiron Corporation is Assistant Secretary and Patent Counsel and in that capacity, I am authorized to act on behalf of Chiron Corporation in executing this document.

2. Chiron Corporation is an assignee of the above-identified patent application as reflected in an assignment recorded with the U.S. Patent and Trademark Office on January 26, 1995 at Reel 7329, Frames 0191-0195.

3. Hybridomas 5D12 and 3C6 were deposited with the American Type Culture collection, 12301 Parklawn Drive, Rockville, Maryland 20852, on May 6, 1993, and were given ATCC Accession Nos. HB 11339 and HB 11340, respectively.

Hybridoma 3A8 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, on January 30, 1996, and was given ATCC Accession No. HB 12024.

4. In accordance with the "Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure," and as per the attached contract with the ATCC, all restrictions on the availability of hybridomas 5D12 (ATCC No. HB 11339), 3C6 (ATCC No. HB 11340) and 3A8 (ATCC No. HB 12024) will be irrevocably removed and these hybridomas will be made permanently available to anyone requesting said hybridomas upon the allowance of the above-identified patent application and the issuance of the patent thereon.

5. If the hybridomas of paragraph 3 should die or be destroyed during the effective term of the deposit, they shall be replaced with cultures of the same.

6. The biological materials deposited as per paragraph 3 above are identical to the biological materials specifically identified in the specification as filed.

7. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. 1001), and may jeopardize the validity of the application or any patent issuing thereon.

Dated: November 19, 1996

Paul B. Savereide, Ph.D.
Registration No. 36,914

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American Type Culture Collection

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Chiron Corporation
Attention: Karen Van Note
4560 Horton Street
Emeryville, CA 94608-2916

Deposited on Behalf of: Chiron Corporation (Case No. #2630.2)

Identification Reference by Depositor:	**ATCC Designation**
Hybridoma 5D12, CMCC 11068	HB 11339
Hybridoma 3C6, CMCC 11067	HB 11340
Hybridoma B7-24-E1G4, CMCC 11060	HB 11341

The deposits were accompanied by: __ a scientific description __ a proposed taxonomic description indicated above.

The deposits were received May 6, 1993 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested May 13, 1993. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon

Date: May 21, 1993

Bobbie A. Brandon, Head, ATCC Patent Depository

cc: Mr. Kenneth Goldman

America Type Culture Collection

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Chiron Corporation
Attention: Karen Van Note
4560 Horton Street
Emeryville, CA 94608-2916

Deposited on Behalf of: Chiron Corporation (Case No. 0925.003)

Identification Reference by Depositor:	ATCC Designation
Hybridoma, 3A8, CMCC #11066	HB 12024

The deposit was accompanied by: __ a scientific description __ a proposed taxonomic description indicated above.

The deposit was received January 24, 1996 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

_X_ We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested January 30, 1996. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Barbara M. Halley

Barbara M. Halley, Administrator, ATCC Patent Depository

Date: February 6, 1996

cc: Paul Savereide

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Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire	
CHIRON CORPORATION	

COMMUNICATION PURSUANT TO ARTICLE 115(2) EPC

Please find enclosed observations by a third party concerning the patentability of the invention of the above-mentioned patent application. That person is not a party to the proceedings before the EPO (Art. 115(1) EPC).

Under Article 115(2) EPC you may comment on the observations.

Formalities Officer
Tel. No. 089/2399 - 7548

A. Ramp

Ramp

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29. März 2004

Munich, March 29, 2004

Our Ref.: AV-2004-264 m2

EP 99104709.3 in the name of Chiron Co. entitled "Antagonistic monoclonal antibodies to human CD40"

Please find enclosed third party observations pursuant to Article 115 EPC.

1. Present claims

In their letter dated 27 January, 2004, the patentee submitted an amended set of claims 1-13.

Claim 1 recites a monoclonal antibody or fragment thereof capable of binding to a human CD40 antigen located on the surface of a human B cell, wherein the binding of the antibody or fragment thereof to the CD40 antigen prevents the growth or differentiation of the B cell.

Claim 2 is directed to the specific monoclonal antibodies 5D12 and 3C6. Claims 3 and 4 are dependent on claim 2 which recites these specific antibodies.

Claim 5 relates to a hybridoma capable of producing a monoclonal antibody having specificity for the CD40 antigen of a human B cell,

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wherein the binding of said monoclonal antibody to a human CD40 antigen expressed on the surface of a human B cell inhibits the growth or differentiation of the B cell.

Claims 6 and 7 recite the same specific antibodies of claim 2.

Claim 10 relates to aCD-40 antigen epitope immunoreactive with an anti-CD-40 monoclonal antibody.

Claims 8, 9 and 11-13 relate to 2nd medical indications using the above monoclonal antibodies or pharmaceuticals comprising these antibodies.

II. The pending claims cannot enjoy the 1st priority date

D1: Journal of Immunological Methods, vol. 152, no. 1, 31 July 1992 (1992-07-31), pages 15-23.(Cited International search report and EPO search report)

In the priority application USSN 07/910222 (filed on July 9, 1992), there is no description regarding the specific feature of the anti-CD40 antibody recited in claims 1 and 5, the specific use of the anti-CD40 antibody recited in claims 8, 9 and 11, the pharmaceutical composition recited in claims 12 and 13, and the specific CD40 antigen epitope recited in claim 10. That is, USSN 07/910222 never discloses an antibody which can prevent the growth or differentiation of B cells.

Regarding the hybridoma claims (claims 5 to 7) and the claim reciting specific antibody clones (claim 2), there is no description of depository number of hybridomas producing monoclonal antibodies 5D12 and 3C6 in USSN 07/910222. Based on the description on page 81 of the present specification, the depository date of hybridoma 3C6 and 5D12 is May 6, 1993. The claimed invention cannot be enabled without the deposition of hybridomas.

Therefore, the effective application date which is the date to judge the novelty and inventive step is the international filing date, July 8, 1 993, or at the earliest, May 28, 1993 (filing date of 3rd priority application).

III. Lack of patentability over de Boer et al (D1)

D1, which was published before May 28, 1993, discloses the anti-CD40 antibodies termed 3A8, 3C6, 5D12 and 5H7 and hybridomas producing these antibodies. D1 also describes that these monoclonal antibodies can be bound to CD40 on B cells (see page 21, left column, lines 12-15; Table 11). CD40 antibodies with the same names and hybridomas producing these same antibodies are described in the present specification.

Present claims 2, 6 and 7 are not novel over D1 since the claimed antibodies and hybridomas are indistinguishable from those disclosed in D1. Claims 1 and 5 also lack novelty over D1, since claims 1 and 5 comprise 5D12 and 3C6 antibodies and hybridomas, respectively. Claims 3 and 4 are anticipated by D1, since humanized antibody and antibody fragments such as Fab' are well-known variations of the monoclonal antibody

At the time of filing the present application, testing B cell proliferation activity was a routine experiment for characterization of the anti-CD40 antibody. Therefore, the person skilled in the art can readily find the specific feature of the 5D12 and 3C6 antibodies, described in D1. Therefore, claims 8, 9, 11, 12 and 13 are readily conceived based on the descriptions of D1.

Regarding claim 10, the third party observer would like to point out that no specific CD40 antigen epitope is shown in the specification.

Joseph Taormino, Ph.D.
Association No. 151

Encl.:
D1: Journal of Immunological Methods, vol. 152, no. 1, 31 July 1992

D 1

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Generation of monoclonal antibodies to human lymphocyte cell surface antigens using insect cells expressing recombinant proteins

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(Received 19 December 1991, revised received 11 February 1992, accepted 12 February 1992)

We have expressed human CD40 and human B7 in insect cells using the baculovirus expression system and have used these insect cells to immunize mice for the generation of monoclonal antibodies. We demonstrate here that specific monoclonal antibodies to human CD40 and human B7 were obtained using this approach. One significant advantage of this method is that immunizing mice with insect cells did not evoke an immune response to human cells and, therefore, EBV-transformed human B cells could be used to screen for specific antibody production by the hybridoma clones.

Key words: Baculovirus expression; Immunization; Monoclonal antibody; Lymphocyte cell surface antigen; Polymerase chain reaction

Introduction

Since the introduction of hybridoma technology by Köhler and Milstein (1975), a large number of monoclonal antibodies have been produced, which specifically bind to molecules expressed on the cell surface of lymphoid cells. These monoclonal antibodies have proven to be powerful tools in immunological research. To obtain monoclonal antibodies against cell surface molecules, mice have been immunized with the cells expressing the molecule of interest. Immunizing mice with whole cells usually results in a strong immune response and, therefore, the use of adjuvants is generally not required. Instead, the question is more how to suppress the immune response to unwanted immunodominant antigens. When mice are immunized with whole cells, antibodies to a large number of different molecules are generated. It is therefore difficult to use the same cells to screen specific antibody production by the hybridoma clones. The cells used for immunization can be used for a pre-screen or in a differential screen, but additional screening assays are needed to determine the specificity of the monoclonal antibodies obtained. Furthermore, when the molecule of interest is expressed at low density, it is likely that the frequency of mouse B cells specific for the antigen will be very low in a strong polyclonal immune response. As a consequence, large numbers of hybridoma clones have to be screened for specific antibody production.

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To circumvent these problems, murine fibroblasts expressing human cell surface antigens have been used to immunize for specific antibody production (DiSanto et al., 1991). When injected into the appropriate mouse strain, the syngeneic fibroblasts should not be immunogenic and the immune response should be focused on the xenogeneic recombinant protein. Here we have used a slightly different approach to address the above-mentioned problems. We have expressed several human cell surface antigens in insect cells and used these insect cells to immunize mice. Using the baculovirus expression system (reviewed by Webb and Summers, 1990), cell surface proteins can be expressed at very high levels in insect cells (Webb et al., 1989). When these cells are used for immunization, the high expression levels of the cloned proteins will increase the chance of obtaining specific antibodies. We demonstrate that immunizing mice with insect cells expressing human CD40 or human B7 resulted in production of specific monoclonal antibodies to these molecules. Furthermore, immunization of mice with insect cells alone did not evoke an immune response to human cells and, therefore, human EBV-transformed B cells could be used for the screening of specific antibodies to human CD40 or human B7.

Materials and methods

Materials

Iscove's modification of Dulbecco's modified Eagle's medium (IMDM) and fetal bovine serum were obtained from JR Biosciences (Lenexa, KS); penicillin and streptomycin were obtained from Irvine (Santa Ana, CA); and polyethylene glycol (mol. wt. 1500) was obtained from Boehringer Mannheim (Indianapolis, IN).

Cell lines

SP2/0 murine myeloma cells, the EBV-transformed B cell line ARC, the T cell lines HSB and CEMM, and Sf9 insect cells were all obtained from the ATCC (Rockville, MD).

Culture media

SP2/0 murine myeloma cells, hybridoma cells, and cell lines were cultured in IMDM supplemented with streptomycin (200 μg/ml), penicillin (200 U/ml) and 10% heat-inactivated fetal bovine serum (complete IMDM). The Sf9 insect cells were cultured in shaker flasks agitated (125–150 rpm) in medium described by Maiorella et al. (1988) supplemented with 0.5% fetal bovine serum.

Antibodies

Anti-(human B7) monoclonal antibody BB-1 (Yokochi et al., 1982) was a gift of Dr. E.A. Clark (University of Washington, Seattle, WA) and was used as purified antibody. Anti-(human CD40) monoclonal antibody G28.5 (Clark and Ledbetter, 1986) was a gift of Dr. J.A. Ledbetter (Oncogen Corporation, Seattle, WA) and was used as purified antibody. Anti-(CD40) monoclonal antibody S2C6 (Paulie et al., 1985) was a gift of Dr. S. Paulie (University of Stockholm, Stockholm, Sweden) and was used as purified antibody. Anti-(human CD26) monoclonal antibody Ta-1 and anti-(CD20) monoclonal antibody B1 were obtained from Coulter (Hialeah, FL). Anti-(CD3) monoclonal antibody OKT3 was obtained from Ortho (Raritan, NJ), and the anti-(LeuM3) monoclonal antibody was obtained from Beckton-Dickinson (San Jose, CA). Anti-(IgM) antibodies coupled to beads (Immunobeads) were obtained from Bio-Rad (Richmond, CA). The monoclonal antibody to the Glu-Glu epitope (Grussenmyer et al., 1985) was used as purified antibody.

PCR cloning of CD40 and B7

RNA was isolated from a population of EBV-transformed human spleen cells as described by Chirgwin et al. (1979). In brief, the cells were washed twice with phosphate buffered saline and lysed in 5 M guanidinium thiocyanate in the presence of 0.7 M 2-mercaptoethanol. The cell lysate was layered on a discontinuous CsCl gradient and centrifuged for 16 h at 26,000 rpm in a Beckman SW28 rotor. The RNA was recovered by dissolving the pellet in diethyl pyrocarbonate-treated H_2O (DEPC treated H_2O). The RNA was precipitated with ethanol once, resuspended in DEPC-treated H_2O, and stored at −70°C.

Total RNA (10 μg/reaction) was converted to cDNA using random hexamer priming in 50 μl

reaction buffer containing 500 U moloney murine leukemia virus reverse transcriptase (BRL, Bethesda, MD), 5 μM random hexamers (Pharmacia, Piscataway, NJ), 1 mM dithiothreitol (DTT), 2'-deoxynucleoside 5'-triphosphates (dNTP mix) (0.5 mM each), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mg/ml bovine serum albumin (BSA). After incubation at 37°C for 1 h, the samples were boiled for 3 min and stored at −70°C. The DNA encoding the CD40 and B7 molecules was generated by polymerase chain reaction (PCR) using primers with restriction sites for cloning (Fig. 1). These primers were based on the published cDNA coding sequences for B7 and CD40 (Freeman et al., 1989; Stamenkovic et al., 1989). For PCR amplification, 1 μl of cDNA was mixed with 1 μl (10 picomol) of a forward primer, 1 μl (10 picomol) of a backward primer, and 47 μl of PCR mix. The PCR mix consisted of 1.25 U Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT), dNTP mix (0.2 mM each), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mg/ml BSA. The 50 μl of PCR mixture was overlaid with 70 μl mineral oil and subjected to 25 cycles of amplification in a Perkin-Elmer/Cetus thermocycler (denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 1.5 min). PCR products obtained after 25 amplification cycles were subcloned in the polylinker of the baculovirus transfer vector pAcC8. Before expression in baculovirus, the DNA inserts of the pAcC8 vector were checked for PCR-induced mutations by sequencing analysis.

Baculovirus expression

Sequences encoding human CD40 and human B7 were recombined into the *Autographa californica* baculovirus (AcNPV) using the transfer vectors pAcCD40 (encoding the full-length CD40 molecule), pAcCD40-ED/Glu (encoding the extracellular domain of CD40), pAcB7 (encoding the full-length B7 molecule), and pAcB7-ED/Glu (encoding the extracellular domain of the B7 molecule). Recombinant viruses were isolated as previously described (Smith et al., 1985; Summers and Smith, 1987). Sf9 cells were infected with recombinant virus (2–10 pfu) at a density of 10^6 cells/ml. For cell surface expression of recombinant proteins, the cells were harvested after 48 h of culture; for the production of secreted recombinant proteins, the cells were harvested after 72 h of culture.

Affinity purification of soluble B7 and soluble CD40

Soluble B7 and soluble CD40, tagged with the Glu-Glu epitope (Glu-Glu-Glu-Glu-Tyr-Met-Pro-Met-Glu) (Grussenmyer et al., 1985), were puri-

Full length B7:

Forward MR81 5'-GCG CTGCAG CATCTGAAGCCATGGGCC-3' (307-324)

Backward MR88 5'-CGC GGTACC TTGCTTCTGCGGAGACTG-3' (1183-1199)

Soluble B7:

Forward MR87 5'-GCG CTGCAG CATCTGAAGCCATGGGCC-3' (307-324)

Backward MR148 5'-GCGC GGTACC TTACTCCATGGGCATTTATTCTTCTTCTTTTGTTATCACGAAAATGCTGTTG-3' (1022-1047)

Full length CD40:

Forward MR108 5'-GCGT AGATCT GGTCTCACCTCGCCATGGTTCG-3' (34-55)

Backward MR111 5'-GCGT CGTACC CCACACTCCTCGGTGTGGTGCATCC-3' (893-913)

Soluble CD40:

Forward MR108 5'-GCGT AGATCT GGTCTCACCTCGCCATGGTTCG-3' (34-55)

Backward MR110 5'-GCGT CGTACC TTACTCCATGGGCATGTATTCTTCTTCTTCATCAGTCTTGTTTGTGCCTGC-3' (515-556)

Fig. 1. Primers used for the amplification of the B7 and CD40 molecules. All primers start with a C-G clamp at the 5' end followed by a restriction site for cloning (shown in bold). The underlined part in the backward primers for the cloning of the soluble forms of B7 and CD40 represents an epitope recognized by a monoclonal antibody used for affinity purification. The numbers in brackets represent the location of the primers on the published cDNAs for CD40 and B7.

fied from concentrated Sf9 insect cell supernatants by affinity chromatography. The affinity matrix consisted of protein G-Sepharose coupled with a monoclonal antibody to the Glu-Glu epitope. Concentrated insect cell supernatant was dialyzed against phosphate-buffered saline (PBS) and loaded onto the column. The column was washed with 5 column volumes of PBS + 0.2% octyl glucoside and 2 mM 2-mercaptoethanol, followed by 5 column volumes of elution buffer (50 mM $NaBO_4$, pH 9.0, 2 mM 2-mercaptoethanol, 0.2% octyl glucoside). The soluble B7 or soluble CD40 tagged with the Glu-Glu epitope was released from the column by adding free Glu-Glu peptide (50 μg/ml in elution buffer). The fractions were neutralized with 0.5 M acetic acid, 0.5 M sodium phosphate, pH 5.5. The eluted proteins were then dialyzed against PBS.

Immunization

Female BALB/c mice were injected intraperitoneally (IP) at day 0 and day 14 with 5×10^6 Sf9 cells infected with AcCD40 virus, AcB7 virus, or AcCd3 virus (control virus). On day 21, 100 μl of serum was obtained to test for the presence of specific antibodies. After a rest period of at least two weeks, the mice received a final injection with 5×10^6 Sf9 cells infected with AcCD40 or AcB7 virus. Three days after this last injection, the spleen cells were used for cell fusion.

Generation of hybridoma clones

Splenocytes from immunized BALB/c mice were fused with SP2/0 murine myeloma cells at a ratio of 10:1 using 50% polyethylene glycol as previously described by De Boer et al. (1988). The fused cells were resuspended in complete IMDM medium supplemented with hypoxanthine (0.1 mM), aminopterin (0.01 mM), thymidine (0.016 mM) and 0.5 ng/ml hIL-6 (Genzyme, Cambridge, MA). The fused cells were then distributed between the wells of 96-well tissue culture plates, so that each well contained one growing hybrid on average. After 10–14 days the supernatants of the hybridoma populations were screened for specific antibody production. For the screening of specific antibody production by the hybridoma clones, the supernatants of 12 wells were pooled and used for fluorescent cell staining on EVB-transformed B cells as described below. Subsequently, the supernatants of the positive pools were tested individually. Positive hybridoma cells were cloned three times by limiting dilution in complete IMDM containing 0.5 ng/ml hIL-6.

Sf9 cell ELISA

Sf9 insect cells infected with recombinant virus were cultured for 48 h in 24-well plates. After removal of the tissue culture medium the plates were incubated for 45 min at room temperature (RT) with 0.25 ml of antibody in PBS with 1% BSA (PBS-BSA). After three washes with PBS-BSA, the plates were incubated for 45 min at RT with 250 μl of a 1/250 dilution of goat anti-(mouse total Ig) immunoglobulins conjugated to horseradish peroxidase (Zymed, South San Francisco, CA) in PBS-BSA. Unbound peroxidase activity was removed by washing five times with PBS-BSA. Bound peroxidase activity was revealed by the addition of an assay mixture prepared by diluting 0.5 ml of 2 mg/ml 3,3',5,5'-tetramethylbenzidine in ethanol to 10 ml with 10 mM Na acetate, 10 mM EDTA buffer (pH 5.0) and adding 0.03% (v/v) H_2O_2. The reaction was stopped after 10 min by adding 100 μl of 1 M H_2SO_4.

Isolation of tonsillar B cells

Tonsillar B lymphocytes were isolated from tonsils obtained from children undergoing tonsillectomy as described by De Groot et al. (1990). Briefly, the tissue was dispersed with scalpel blades, phagocytic cells and NK cells were depleted by treatment with 5 mM L-leucine methyl ester, and T cells were removed by one cycle of rosetting with sheep erythrocytes treated with 2-aminoethyl isothiouronium bromide.

Fluorescent cell staining

Cells (10^6/sample) were incubated in 100 μl primary antibody (10 μg/ml in PBS-BSA or Hanks' balanced salt solution (HBSS) supplemented with 1% BSA and 0.05% sodium azide) for 20 min at 4°C. After three washes with PBS-BSA or HBSS-BSA, the cells were incubated in 100 μl FITC-labeled $F(ab')_2$ fragments of goat anti-(mouse IgG) antibodies (Jackson, West

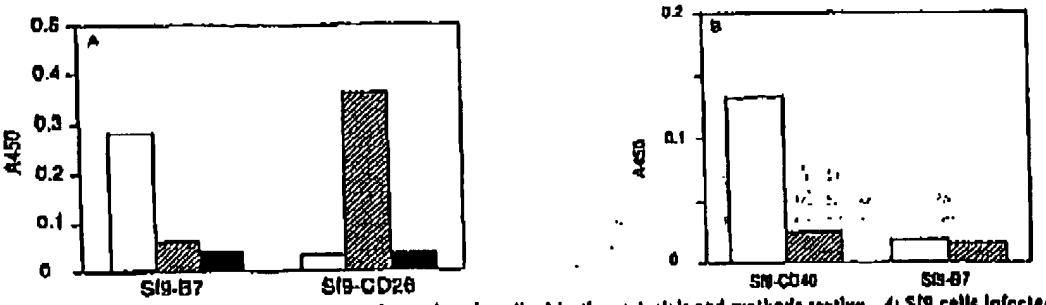

Fig. 2. ELISA assays on live Sf9 cells were performed as described in the materials and methods section. *A*: Sf9 cells infected with pAcB7 and pAcCD26 were cultured for 48 h in 24-well plates. The antibodies used in the ELISA were: B7–24, anti-(B7) (open bars), Ta-1, anti-(CD26) (hatched bars) and no primary antibody (gray bars). *B*: Sf9 cells infected with pAcB7 and pAcCD40 were cultured for 48 h in 24-well plates. The antibodies used in the ELISA were: S2C6, anti-(CD40) (open bars) and no primary antibody (hatched bars).

Grove, PA) for 20 min at 4°C. After three washes with PBS-BSA or HBSS-BSA and one wash with PBS, the cells were resuspended in 0.5 ml PBS. Analyses were performed with a FACSCAN V (Becton Dickinson, San Jose, CA). For analysis of cultured tonsillar B cells, propidium iodine was used to exclude dead cells.

Results

Full length cDNAs encoding human B7 and human CD40 were generated by PCR using primers with restriction sites for cloning. The template for PCR amplification was cDNA generated from EBV-transformed human spleen B cell RNA. The expression of the recombinant molecules on the cell surface of the Sf9 cells was tested using an ELISA system. Fig. 2*a* shows that the anti-(B7) monoclonal antibody BB-1 reacted only with Sf9 cells infected with AcB7 virus, but not with Sf9 cells expressing human CD26. In contrast, the anti-(CD26) monoclonal antibody Ta-1 reacted only with the Sf9 cells expressing CD26, but not B7. Similar results were obtained with Sf9 cells expressing CD40. Fig. 2*b* shows

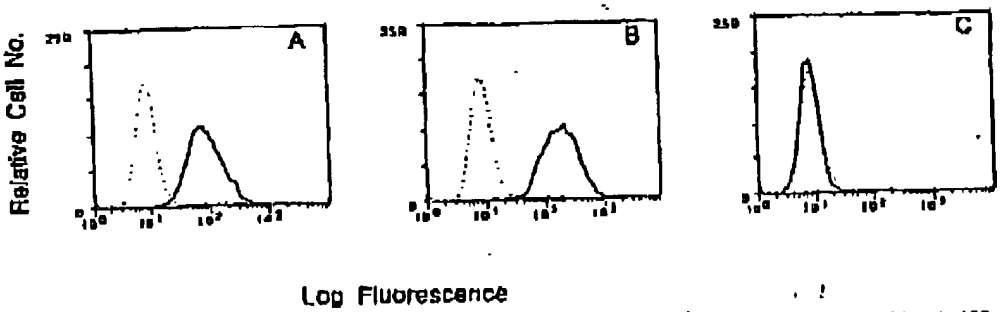

Fig. 3. Fluorescent cell staining of ARC cells as described in the materials and methods section. *A*: staining with a 1/100 dilution of serum from a mouse immunized with B7 expressing SF-9 cells (solid line) or a 1/100 dilution of normal mouse serum (dotted line). *B*: staining with a 1/100 dilution of serum from a mouse immunized with CD40 expressing Sf9 cells (solid line) or a 1/100 dilution of normal mouse serum (dotted line). *C*: staining with a 1/100 dilution of serum from a mouse immunized with control Sf9 cells (solid line) or a 1/100 dilution of normal mouse serum (dotted line).

that the anti-(CD40) monoclonal antibody S2C6 reacted only with Sf9 cells expressing CD40, but not with Sf9 cells expressing B7.

For the generation of monoclonal antibodies to CD40 and B7, mice were immunized with the Sf9 cells expressing these molecules on the cell surface. 1 week after the second immunization, the mice were bled and the sera were analyzed for the presence of specific antibodies using fluorescent cell staining of EBV-transformed B cells. Fig. 3 shows that mice immunized with Sf9 cells expressing CD40 or B7 had a serum titer against EBV-transformed B cell line ARC, which is positive for both CD40 and B7. In contrast, mice which were immunized with control Sf9 cells showed no reactivity with the ARC cells.

Since the mice immunized with Sf9 cells expressing CD40 or B7 had a serum titer when tested on ARC cells, we gave one mouse a final booster injection with CD40 expressing Sf9 cells and one with B7 expressing Sf9 cells. 3 days after the booster injection, the spleens were removed and the splenocytes were fused with SP2/0 murine myeloma cells. Table I gives a summary of the fusion data. After the CD40 fusion, only half of the cells were seeded in 480 wells. This resulted in 351 wells with hybridoma growth. After

TABLE I

SUMMARY OF FUSION DATA FOR THE GENERATION OF MONOCLONAL ANTIBODIES TO HUMAN CD40 AND HUMAN B7

Fusion	Anti-CD40	Anti-B7
no. of wells seeded after fusion	480 [a]	960
no. of wells with hybridoma growth	351	317
no. of positive wells [b]	4	1
Frequency of positive wells [c]	1.15	0.31

[a] Only half of the cells obtained after fusion were analyzed.
[b] As determined by FACS analysis described in the materials and methods section.
[c] The frequency of positive wells is defined as the number of positive wells divided by the total number of wells with hybridoma growth, multiplied by 100.

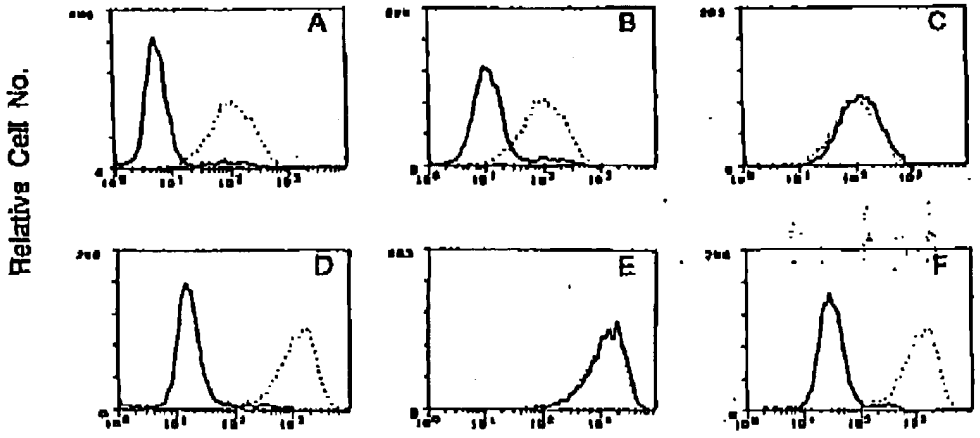

Fig. 4. Fluorescent cell staining of ARC EBV-transformed B cells. Staining with anti-(B7) and anti-(CD40) monoclonal antibodies in the presence and absence of soluble B7 and soluble CD40. The antibodies and the soluble B7, soluble CD40, or controls were preincubated at RT for 20 min before addition to the ARC cells. *A*: staining with B7–24 (dotted line) or secondary antibody only (solid line). *B*: staining with B7–24 alone (dotted line) or B7–24 preincubated with soluble B7 (solid line). *C*: staining with B7–24 alone (dotted line) or B7–24 preincubated with soluble CD40. *D*: staining with CD40–3A8 (dotted line) or second antibody only (solid line). *E*: staining with CD40–3A8 alone (dotted line) or CD40–3A8 preincubated with soluble B7 (solid line). *F*: staining with CD40–3A8 alone (dotted line) or preincubated with soluble CD40.

the B7 fusion, the cells were distributed in 960 wells and this fusion yielded 312 wells with hybridoma growth. 14 days after the fusions, supernatants of 12 wells were pooled and the pools were tested for the presence of antibodies reactive with ARC cells. FACS analysis revealed that four pools from the CD40 fusion and one pool from the B7 fusion were reactive with ARC cells. When individual supernatants from the positive pools were retested, four wells reactive with CD40 and one well reactive with B7 were identified. The cells from these positive wells were cloned by limiting dilution and, after three rounds, four stable anti-(CD40) hybridoma clones (CD40–3A8, CD40–3C6, CD40–5D12 and CD40–5H7) and 1 stable anti-(B7) hybridoma clone (B7–24) were established.

All five hybridoma clones were reactive with ARC cells and other EBV-transformed B cell lines, but not with T cell lines HSB and CEMM (results not shown). However, the specificity of these monoclonal antibodies had yet to be proven. Therefore, we performed competition experiments in which soluble forms of CD40 and B7 competed with CD40 and B7 expressed on the ARC cells for the binding of the monoclonal antibodies. Hybridoma supernatants were pre-incubated with soluble forms of CD40 and B7. Subsequently, the mixtures were added to ARC cells for fluorescent cell staining. Fig. 4 shows that soluble B7, but not soluble CD40, could block the binding of anti-(B7) monoclonal antibody B7–24 to ARC cells. Conversely, soluble CD40, but not soluble B7, could block the binding of anti-(CD40) monoclonal antibody CD40–3A8 to ARC cells. Similar results were obtained with the other three anti-(CD40) monoclonal antibodies. Furthermore, the effects of soluble CD40 on the anti-(CD40) monoclonal antibodies and the effect of soluble B7 on the anti-(B7) monoclonal antibody was concentration-dependent. Decreasing the amount of soluble protein resulted in decreased blocking of antibody binding to ARC cells (results not shown).

For further analysis of the anti-(CD40) and anti-(B7) monoclonal antibodies, we tested their ability to bind to tonsillar B cells. Table II shows that 89–95% of freshly isolated tonsillar B cells stained positive with the four anti-(CD40) monoclonal antibodies. About the same percentage of cells was positive with anti-(CD40) monoclonal antibody G28.5. Table III shows that 12–17% of freshly isolated tonsillar B cells stained positive with anti-(B7) monoclonal antibody B7–24. However, when tonsillar B cells were cultured for 5 days in the presence of immobilized anti-(IgM) antibodies and IL-2, the percentage of cells positive for B7–24 increased up to about 25% (results not shown). Furthermore, when tonsillar B cells were stimulated with anti-(IgM) antibodies and IL-2, not only did the number of B cells positive for B7–24 increase, but there was also a significant increase in the amount of fluorescent staining per cell, indicating that the expression of B7 was increased after stimulation (results not shown).

TABLE II

BINDING OF ANTI-(CD40) MONOCLONAL ANTIBODIES TO HIGHLY ENRICHED TONSILLAR B CELLS

Antibody	Specificity	Percentage of positive cells [a]
OKT3	CD3	2.1
LeuM3	LeuM3	2.4
B1	CD20	88.0
G28.5	CD40	92.1
CD40-5H7	CD40	93.7
CD40-5D12	CD40	93.0
CD40-3C6	CD40	88.9
CD40-3A8	CD40	93.3

[a] The percentage of positive tonsillar cells was measured by fluorescent cell staining as described in the materials and methods section.

TABLE III

BINDING OF ANTI-(B7) MONOCLONAL ANTIBODY B7-24 TO HIGHLY ENRICHED TONSILLAR B CELLS

Antibody	Specificity	Percentage of positive cells [a]	
		Donor 1	Donor 2
OKT3	CD3	6.0	2.1
B1	CD20	74.0	88.0
B7-24	B7	12.0	16.8

[a] The percentage of positive tonsillar cells was measured by fluorescent cell staining as described in the materials and methods section.

Discussion

For the production of monoclonal antibodies one would generally like to immunize mice with purified material. However, purification of membrane antigens requires specialized and complex techniques and, furthermore, extraction from the membrane may alter the structure of the molecule. In addition, solubilization of proteins often decreases their immunogenicity. Therefore, most monoclonal antibodies to cell surface antigens have been obtained after immunization of mice with whole cells or membrane fractions. In many cases, specific lymphocyte subsets have been injected into mice resulting in panels of monoclonal antibodies. These antibodies have been used to isolate and characterize the antigen that they bound. When mice are immunized with whole cells, antibodies to a large number of different molecules are generated. It is therefore difficult to use the same cells for the screening of specific antibody production by the hybridoma clones.

To circumvent the above-mentioned problem, we have expressed several known human cell surface proteins in Sf9 insect cells and used these insect cells to immunize mice. Since the introduction of PCR technology (Saiki et al., 1985, 1988), it has become relatively simple to clone the cDNAs for proteins whose coding DNA sequence has been published. One can use PCR primers spanning the complete coding region only, and incorporate restriction sites in these primers to facilitate cloning into expression vectors.

It has been shown that human intracellular, secreted, and transmembrane proteins can be expressed at high levels in Sf9 insect cells when inserted under the regulation of the nonessential baculovirus gene for the polyhedrin protein (reviewed by Luckow and Summers, 1988). Since recombinant proteins can be expressed at very high levels on the cell surface of insect cells, a large proportion of the immune response after injection of these cells will be directed against the recombinant protein. We found that only 2 i.p. injections with 5×10^6 Sf9 cells expressing human CD40 or human B7 gave good serum titers against these antigens. Furthermore, the insect cells themselves did not evoke an immune response cross-reactive with human cells. This enabled us to use EBV-transformed B cells for the screening of specific antibody production by the hybridoma clones, with minimal risk of obtaining false positives. All of the positive primary wells obtained were in fact specific for the antigen that was used for immunization.

Since we used EBV-transformed B cells for the screening of the primary hybridoma supernatants and during the limiting dilution cloning, the specificity of the monoclonal antibodies remained to be demonstrated. Several lines of evidence presented here suggest that we have indeed generated four anti-(CD40) and one anti-(B7) monoclonal antibodies. First, there is positive staining with these antibodies on EBV-transformed B cells, but not on T cell lines. Secondly, the competition experiment shown in Fig. 3 demonstrated that the anti-(CD40) antibodies bound to a soluble form of CD40 but not to a soluble form of B7. Likewise, the anti-(B7) antibody only bound to the soluble B7 molecule. Finally, the staining with the antibodies on tonsillar B cells as reported in Tables II and III is in agreement with what has been published for other anti-(B7) and anti-(CD40) monoclonal antibodies (Yokochi et al., 1982; Paulie et al., 1985; Freedman et al., 1987; Valle et al., 1990). Although we, like Valle et al. (1990), found a smaller increase in the number of tonsillar B cells expressing B7 after stimulation with immobilized anti-(IgM) antibodies than has been reported by Freedman et al. (1987), this could be due to differences in the experimental procedures used.

It should be mentioned that the fusion data described in this paper, in particular the frequency of positive clones in the B7 fusion, is not optimized. It is therefore difficult to draw conclusions about the efficiency of obtaining specific hybridoma clones using this system. Both fusions were the first and, so far, the only ones that we performed with the mice that were immunized, yet both fusions yielded specific monoclonal antibodies. We therefore feel that the method for the generation of monoclonal antibodies to cell surface antigens described in this paper is relatively fast and convenient.

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